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Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Colletotrichum gloeosporioides* isolated from *Lannea corammendalica*

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Abstract: An endophytic fungus isolated from *Lannea corammendalica* displayed considerable antimicrobial activity. The fungus was identified as *Colletotrichum gloeosporioides* based on morphological features. The metabolite showed more activity against gram positive than gram negative bacterial pathogens. Antimicrobial activity against *Staphylococcus aureus* was highest with zone of inhibition 25 mm. The fungal biomass was extracted for intracellular metabolites by using ethyl acetate as solvent. The crude extract was filtered, and the filtrate was dried under vacuum at 40°C. The filtrate was analyzed for secondary metabolites. The metabolite was characterized and identified by Gas-Chromatography Mass-spectrophotometry (GC-MS) analysis due to its volatile nature. The main components were 9- octadecenamide, hexadecanamide, diethyl pythalate,2-methyl-3-methyl-3-hexene, 3-ethyl-2,4-dimethyi-pentane. The metabolite produced by the endophytic fungus could be an alternative source of antimicrobial agents against clinical pathogens
Key word: Endophytic fungi, secondary metabolite, antimicrobial activity

Introduction:

The emergence of antibiotics resistance developed in bacterial pathogens and the current increase in the number of new diseases and pathogens demands constant search for new antimicrobial compounds with novel mode of action. Endophytic fungi are fungal microorganisms which asymptomatically inhabit plant tissues and have been isolated from many species of woody plants and grasses [1]. These fungal endophytes have been recognized as a repository of novel secondary metabolites, which have antibiotic, antimycotic, immuno suppressive, and anticancer activity[2].Antimicrobial metabolites (Antibiotics) can be defined as low-molecular-weight organic compounds made by microorganisms that are active at low concentrations against other microorganisms, not required for its growth, produced as an adaptation for specific functions in nature, and are the most bioactive natural products isolated from endophytes[3].Endophytes are believed to carry out a resistance mechanism to overcome pathogenic invasion by producing secondary metabolites bearing antimicrobial activity. It is believed that screening for antimicrobial compounds from endophytes is a promising way to overcome the increasing threat of drug resistant microbes of human and plant pathogen [4].Exploitation of novel classes of antimicrobial metabolites is increasingly noticeable over recent years.A considerable body of research has investigated the diversity,ecological role, secondary metabolites and bioactivityof the endophytic fungi isolated from various medicinal plants [3].

One such plant studied is *Lannea coromandelica.* which is commonly known as "The Indian Ash Tree" . It is a deciduous tree which grows up to 14 metres high. It belongs to the family Anacardiaceae. The bark of the tree and leaves are used as traditional medicine to cure sprains, bruises, skin eruptions, heart diseases, dysentery, mouth sores., toothache and diabetes [5]

In this study an attempt was made to investigate the fungal endophytes producing bioactive metabolites and identifiy the antimicrobial metabolite by Gas-Chromatography Mass- Spectrophotometry (GC-MS).

Experimental

Isolation and identification of the fungus :

The healthy plant were washed in running tap water The leaf segments were cut into 2 mm^2 segments and were surface sterilized by The leaf segments were cut into 2 mm^2 segments and were surface sterilized by sequentially plunging into 70% ethanol for 5 seconds, followed by 4% sodium hypochlorite for 90 seconds and then rinsed with sterile water for 10 seconds. The excess moisture was blotted in a sterile filter paper .In each petri dish, 4-5 segments were placed on Potato Dextrose Agar (PDA) supplemented with antibiotics streptomycin (100 µg/mL concentration), (Sigma, St. Louis, MO, USA) to suppress the growth of bacteria After inoculation the petri dishes were sealed with parafilm and incubated at 27°C ± 10°C for 7 days .Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the segment surface. The plates were examined for fungal growth, the fungus grown out from explant were sub cultured in PDA plates. Later the purified endophytic fungi were transferred to PDA slants separately and were kept at 4°C after being cultured at 28°C for 7 days.

The endophytic fungi grew out from the leaf and bark were identified on the basis of cultural characteristics like texture, colour, surface, elevation reverse side and margin, and the morphology of fruiting bodies and spores ,using standard monographs [6]

Cultivation and metabolite extraction:

The fungus was cultivated on potato dextrose broth by placing agar blocks of pure culture (3 mm in diameter) of actively growing culture in 500 ml Erlenmeyer flask containing 200 ml of the medium. The flask was incubated in BOD shaking incubator for 15 days at 24±2°C with periodic shaking at 150 rpm. The fermentation broth of the endophyte was filtered through cheesecloth to remove the mycelial mats. The filtrate was extracted thrice with ethyl acetate at room temperature. The pooled extract after drying over anhydrous MgSO4, was evaporated in a rotary vacuum evaporator. The crude extract was then dissolved in dimethyl sulphoxide (DMSO, Merck) to obtain different concentrations for antimicrobial bioassay [7].

Determination of antimicrobial activity:

Ethyl acetate extracts from endophytic fungus was screened for antibacterial activity against three gram positive bacteria namely *Bacillus subtilis, Staphylococcus epidermidis* and *Staphylococcus aureus,* three gram negative bacteria namely *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Escherichia coli* .About 1ml of the inoculums of the test pathogen was spread into Nutrient Agar plates. A 5mm well was made in each corner of the plate with equal distance using a sterile cork borer. The ethyl acetate extract with different concentrations at 25, 50, 75 and 100µg compared with standard antibiotic were placed in their respective well and the plates were incubated at 37ºC for 48h. DMSO was used as a control. After the incubation, the inhibition zone around the well was recorded and expressed as millimetre **[8].**

Separation of the active metabolite:

1 µl of the ethyl acetate extract of *Colletotrichum gloeosporioides* was employed for GC/MS analysis. GC-MS analysis was carried out on a GC QP 2010 [SHIMADZU] comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 × 0.25 mm ID ×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) was used as carrier gas at a constant flow of 1ml/min and an injection volume of 0.5 EI was employed (split ratio of 10:1) injector temperature 240°C; ion-source temperature 200°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C/min, then 5°C/min to 280°C/min, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Identification of components:

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

Result and discussion:

The fungus, isolated as endophyte from healthy leaves of *Lannea coramendalica grew* as white coloured colony which gradually turned greyish white as the culture grew older. Aerial mycelium slightly flocculose with orange conidial pustules evident at the centre of the colony. Reverse of colony appeared smoky grey in colour on PDA medium. The mycelium was hyaline, brown or both .On the basis of morphological characters of the fungus it was identified and confirmed as *Colletotrichum gloeosporioides.* In this study *Colletotrichum gloeosporioides* have been reported as endophyte. Various studies have shown that *Colletotrichum gloeosporioides* has been isolated as endophytes from a wide host [9] with strong antimicrobial activity against various strain[10]. A new antimicrobial metabolite, colletotricacid has been isolated from endophytic fungus *Colletotrichum gloeosporioides* from *Artemisia Mongolic*[11].

The screening of endophytic fungi crude extracts for their antimicrobial activity was demonstrated for 8%-92% of endophytic extracts in other studies[12]. against tested pathogens such as *Staphylococcus aureus, Bacillus subtilis, Saccharomyces cerevisiae* and*Alter-naria*sp., *etc.* The crude metabolite was extracted from fermentation broth of the fungus by solvent extraction procedure. The metabolite exhibited strong to moderate antimicrobial activity against all the test pathogens (Table 1). To assess the magnitude of antimicrobial action, the metabolites were co-assayed with reference antibiotics i.e., tetracycline as

antibacterial The metabolite showed highest zone of inhibition against *Staphylococcus aureus* (25 mm) followed by *Staphylococcus epidermidis* 20 mm) whose antibacterial activity was almost similar to that of the positive control, tetracycline. Among bacterial pathogens, the metabolite showed lowest activity against *Klebsiella pneumoniae* (10 mm). The activity of the metabolite was found to be similar to that of reference antibacterial agent, tetracycline.

Table-1Antimicrobial activity of ethyl acetate extract of *Colletotrichumgloeosporioides*

Test microorganism	Zone of inhibition (mm)				
	25µg/ml	**50µg/ml**	**75µg/ml**	**100µg/ml**	**Tetracycline 20µg/ml**
Staphylococcus epidermidis	13	16	20	20	21
Staphylococcus aureus	15	19	20	25	20
Bacillus subtilis	13	17	19	21	20
Klebsiellapneumoniae	-	5	7	10	18
Escherichia coli	5	10	12	15	19
Pseudomonas aeruginosa	6	9	11	13	18

Similar results were shown by the crude metabolites of *Colletotrichumgloeosporioides* isolated from the medicinal plant *Phlogacanthusthyrsiflorus*Nees, which displayed effective inhibition against the test bacterial strain. [13]. This result was consistent to the observation that Gram positive bacteria are more susceptible towards plants and endophyte extracts as compared to Gram negative bacteria [14] These differences may be attributed to fact that the cell wall in Gram positive bacteria is of a single layer, whereas the Gram negative cell wall is multilayered structure [15], the passage of the active compound through the Gram negative cell wall may be inhibited.

In this study ethyl acetate was used as extraction solvent since it is the most efficient method for obtainment of fungal secondary metabolites [16].The ethyl acetate extract were characterized and identified by GC-MS analysis In our study the metabolite produced by *Colletotrichum gloeosporioides was* identified as volatile hydrocarbons. (Fig-1)So far, studies have reported a large number of antimicrobial compounds isolated from endophytes, belonging to several structural classes such as alkaloids, peptides, steroids, terpenoids, phenols, quinines, and flavonoids [17]. The major identified compounds are presented in (Table 2). The main components were 9-octadecenamide, hexadecanamide, Diethyl pythalate,2-methyl-3-methyl-3-hexene, 3-ethyl-2,4-dimethyi-pentane.Volatile hydrocarbons metabolites have also been reported in some endophytic fungi with antimicrobial activity against human and plant pathogenic bacteria and fungi [18].

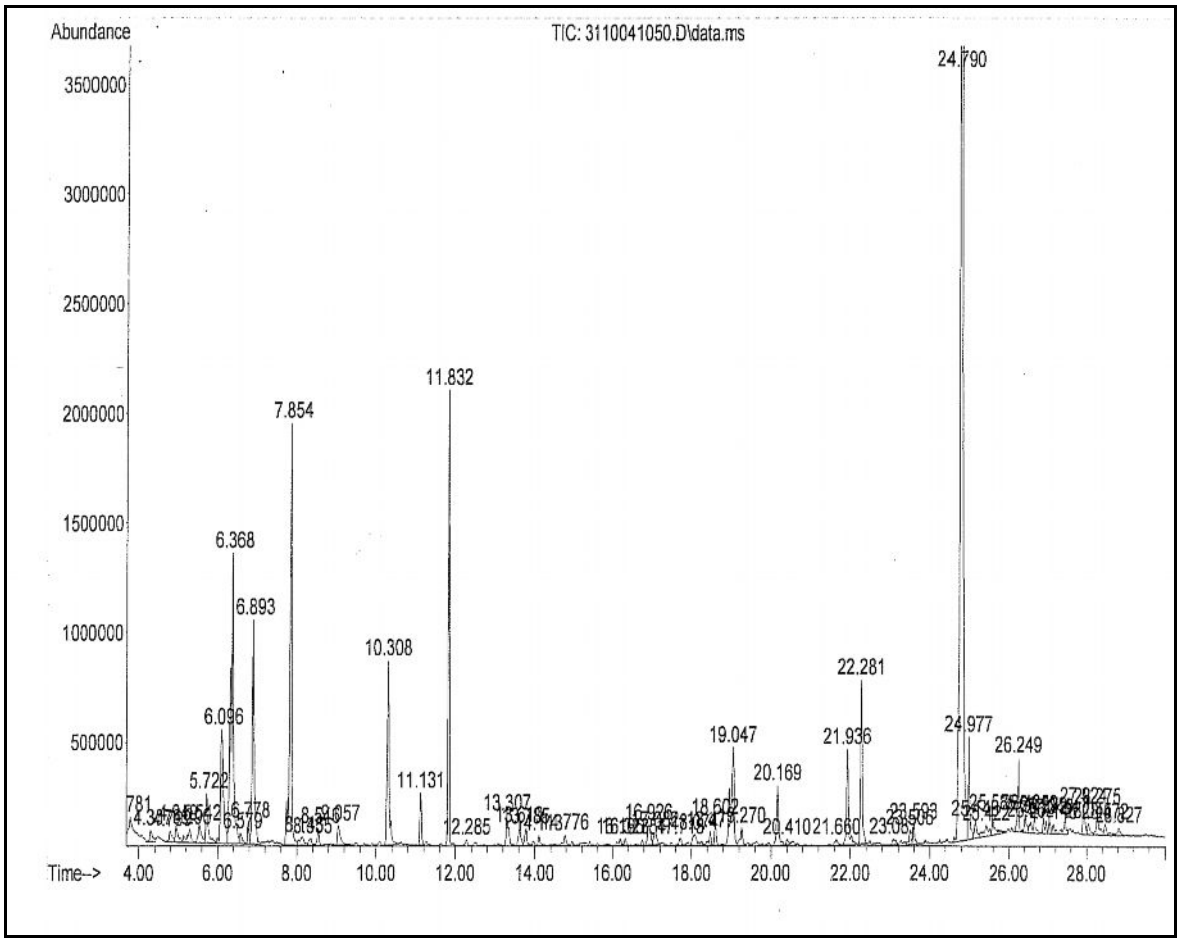

Fig-1 GC-MS spectrum of ethyl acetate extract of *Colletotrichum gloeosporioides*

Table-2 GC-MS analytical report of ethyl acetate extract of *Colletotrichum gloeosporioides*

S.no	Retention time	Area %	Chemical name
1	5.722	0.98	2-hexanone
2	6.096	2.88	Ethanamine
3	6.368	6.79	3-ethyl-2,4-dimethyi-pentane
4	6.893	3.86	4H-1,2,4-Triazole
5	7.854	7.20	2-methyl-3-methyl-3-hexene
6	10.308	3.17	2-methyl-2-propyl oxirane
7	11.131	0.68	1H-2-benzopyran-1-one
8	11.832	5.02	Diethyl pythalate
9	19.047	2.86	Decanoic acid
10	20.169	1.13	2-nitro-4-heptanol
11	21.936	1.68	7-nonenamide
12	22.281	2.30	Hexadecanamide
13	24.790	43.72	9-octadecenamide
14	24.977	1.20	Hexadecanamide
15	26.249	0.82	1,2-Benzenedicarboxylic acid

The major compounds identified are lipophilic compounds and these compounds may attribute the antimicrobial activity. Studies have shown that presence of lipophilic com pounds (mainly long chain fatty acids) can be closely related to its antibacterial activity, as these compounds easily pass through lipid coating of bacteria and coagulate cellular proteins [19].Also presence of diethylphthalate contributes to antimicrobial activity which induce cells to produce superoxide anion $\bullet O_2$ and H_2O_2among others; this results in an accumulation of the lipid peroxides, attacking the polyunsaturated fatty acid in the cell membranes of the organisms leading to cell death. [20].More over octadecadienamide, inhibits the activity of lipoxygenases and

cyclooxygenases, which are important enzymes in fatty acid metabolism,hence causes disruption of cell metabolism finally leading to death of microbes.

Conclusion:

In conclusion we can report that a fungal endophyte *Colletotrichum gloeosporioides* has been isolated from *Lannea corammendalica* which hasgood antimicrobial activity. A further study performed to identifiy the bioactive compounds present in the extract indicates the presence of 9- octadecenamide, hexadecanamide, Diethyl pythalate,2-methyl-3-methyl-3-hexene showing potent antimicrobial activity. Further exploration of the function of thesecompound will facilitate a better understanding towards developing these bioactive compounds as an effective antimicrobial agent.

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